Reprinted from *The Journal of General Physiology*, July, 1966
Volume 49, Number 6, Part 2, pp. 263–304
Printed in United States of America

Problems of an RNA Genome Operating in a DNA-Dominated Biological Universe

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A. INTRODUCTION

The existence of viruses which use RNA rather than DNA to store genetic information poses an interesting challenge to those concerned with the nature and function of genetic material. Questions are raised concerning replication, transcription, and translation involving a gene which cannot be distinguished from its message. These, and related problems posed by RNA genomes, must be resolved before our understanding of gene action and replication can be either satisfying or complete.

We began our study of RNA replication soon after the announcement some years ago by Loeb and Zinder (1) that they had found an RNA bacteriophage. This discovery ultimately made possible the use of the whole armamentarium of techniques that had accumulated as a result of the pioneering efforts of Luria, Delbrück, Hershey, and others with the *E. coli*–T-phage system.

We will trace here our own efforts at understanding the functioning of RNA genomes. In the process an attempt will be made to provide a background of the thinking that went into the experiments to be described. Because of the dominance of serendipity in biological and biochemical research, it is not often either informative or useful to record the reasons for a particular set of experiments; noting the outcome is usually sufficient. It is possible that the present instance may represent an exception.

B. THE PROBLEM OF COMMUNICATION BETWEEN AN RNA VIRUS AND ITS HOST CELL

We start with the fact that all organisms which use RNA as their genomes are mandatory intracellular parasites. They must, therefore, carry out a major part of their life cycle in cells which use DNA as genetic material and RNA as genetic messages. On entry, the viral RNA is faced with the problem of inserting itself into the cellular information flow pattern in order to communicate its own instructions to the synthesizing machinery. A possibility one might entertain centers on whether an RNA virus employs the DNA to RNA to

protein pathway of information flow. This could occur either because the DNA of the host already contains a sequence homologous to the viral RNA, (i.e., the "escaped genetic message" hypothesis), or because such DNA sequences are generated subsequent to infection by reversal of the DNA-dependent RNA synthesizing reaction (2). It is clear that a decision on the existence or nonexistence of homology between viral RNA and the host DNA is a necessary prelude to further experiments designed to delineate the molecular life history of an RNA genome.

The search for homology between two nucleic acid molecules rests essentially on a comparison of sequence. In practice what can readily be detected is sequence complementarity. Experimentally, then, the question we seek to answer may be posed as follows: Does the DNA found in the host cell contain, either before or after infection, a sequence complementary to the nucleic acid of an RNA virus which can infect it?

To answer questions of this nature, recourse was had to the specific hybridization test of Hall and Spiegelman (3) which employs isotopic labeling and centrifugation in CsCl gradients in preparative rotors combined with the subsequently developed (4) use of RNAse to eliminate "noise." Our earlier success with ribosomal and sRNA complexes with DNA (4–6) encouraged us to make an analogous attempt (7) with the MS-2 RNA bacteriophage. The molecular weight of the viral genome is 1×10^6 daltons which means that we are looking for a complementary stretch of DNA equivalent to about 0.036% of the genome of *E. coli*, a comparatively small number. However, the sensitivity of the hybridization had already been brought to the level required in the experiments which identified the DNA complements of sRNA (6).

If at all possible, it is important to arrange experiments of this sort so that a negative outcome is interpretable with as much certainty as a positive result. An element of assurance was therefore introduced into these experiments as an internal control. It happens that the 23S ribosomal RNA of *E. coli* and the RNA of the MS-2 bacteriophage are comparable in molecular weights and base composition and a correspondence heat in induced hyperchromicity (7). Since the responses of these two molecules to heat destruction of their secondary structures are very similar, their behavior during the annealing process of hybridization should be comparable. Consequently, in addition to serving as a test for the sensitivity of hybrid detection, ribosomal RNA can also be used to monitor the adequacy of the hybridization test conditions. Thus, in an experiment which yields hybrids with ribosomal RNA the absence of such complexes with viral RNA can be accepted with comparative confidence as evidence that there is no homology between viral RNA and cellular DNA.

In order to make the experiment even more certain, the double label technique was used in which the 23S RNA was labeled with ^3H and the viral RNA with ^{32}P. Both could then be included in the same reaction mixture and an examination made for complex formation with either one or both of the two constituents.

Results of a number of experiments are illustrated in Fig. 1 in which 1 *a* shows the usual hybrid structure that results when ribosomal RNA is incubated with homologous single-stranded DNA. Fig. 1 *b* describes the completely negative outcome obtained when viral RNA is substituted for ribosomal RNA in the reaction mixture. The results obtained when the DNA is challenged simultaneously with ^3H-23S RNA and ^{32}P-MS-2 RNA are shown in Fig 1 *c*. Here one sees that the ^3H-ribosomal RNA forms a hybrid structure and the ^{32}P-labeled viral RNA is completely excluded. Comparison between Figs. 1 *a* and 1 *c* demonstrates that the presence of viral RNA has no detectable influence on the capacity of ribosomal RNA to complex with its com-

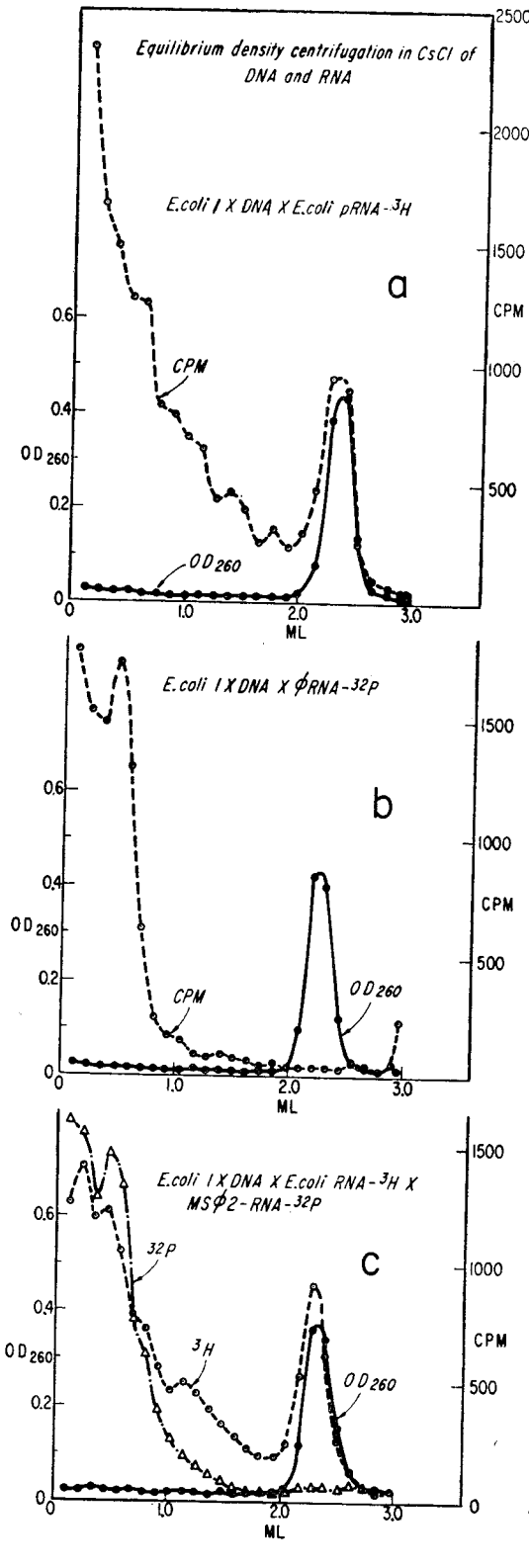

FIG. 1. Equilibrium density gradient centrifugation of incubated mixtures of RNA and DNA. In all cases the peak in optical density identifies the position of DNA in density gradient which decreases from left to right. In addition to 50 μg of heat-denatured DNA of *E. coli*, the annealing mixtures contained the following: (*a*) 2 μg of tritium-(^3H) labeled ribosomal RNA (40,000 CPM per μg); (*b*) 2 μg of ^{32}P-labeled viral RNA (85,000 CPM per μg); (*c*) a mixture of *a* and *b*. The incubation solution was 0.3 M in NaCl, and 0.05 M in phosphate at pH 6 to 8; the total volume was 0.5 ml. The mixtures were slow cooled from 55° to 30°C over a period of approximately 17 hr. The density was then adjusted to 1.720 with CsCl and the final volume was adjusted to 3.0 ml. The resulting samples were centrifuged for 72 hr at 33,000 RPM at 25°C in the SW39 rotor of the Spinco model L ultracentrifuge. At the end, fractions of 0.12 ml were collected from the bottom of the tubes, diluted, and analyzed for optical density at 260 mμ and for radioactivity. Radioactivity of the acid-insoluble fraction was assayed on aliquots, washed and dried on Millipore membranes, and counted in the Packard liquid scintillation spectrometer which measures simultaneously ^3H and ^{32}P. Optical density at 260 mμ was measured on the whole sample; the radioactivity was assayed on a fraction. The activity given must be multiplied by 5.6 to convert to the activity of the total sample (Doi and Spiegelman, 7).

plementary sequence. Fig. 1 *b* shows that the negative finding of Fig. 1 *a* cannot be explained by an inhibitory effect of ribosomal RNA on the ability of viral RNA to hybridize.

Similar experiments with identical results have been carried out with DNA from both infected and noninfected cells and under a variety of other conditions. In the experiments summarized the specific activity of viral RNA used was such that if one sequence complementary to viral RNA existed per genome, 1,800 CPM would have been observed in the DNA density region of the gradient of Fig. 1, and none was found.

It is evident that neither before nor after infection can one find sequences in the DNA which are complementary to the viral RNA. In evaluating these negative findings, several features of these experiments must be borne in mind. First, the hybridization process was monitored internally. Second, the relative specific activity was such that the sensitivity per strand of detecting hybrids with viral RNA was ten times that possible in the case of ribosomal RNA. Finally, the procedure was sensitive enough to have yielded an unequivocal positive result if each genome contained complementary DNA stretches equivalent in length to one-tenth of a viral RNA.

The negative outcome of the hybridization test with DNA from both infected and noninfected cells leads to a numberof interesting conclusions. It obviously argues against the occasionally attractive speculation that RNA viruses might represent escaped genetic messages of the host. It should be noted that two variant possibilities are not eliminated; one suggesting that the originating DNA sequence has been lost from the host genome and the other that the virus particle has evolved away from its original host. Further, the experiments do not support the idea that the viral RNA induces in the cell the formation of a complementary DNA copy. DNA is apparently not an informed intermediary necessary for the synthesis of virus-specific macromolecules. The data do suggest that RNA viruses do not employ the DNA to RNA path of information transfer. This in turn implies that these viruses must have evolved a mechanism of generating RNA copies from RNA. We would then predict the existence of an enzymatic mechanism involving an RNA-dependent RNA polymerase which we have named (7 *a*) "replicase" for purposes of brevity and alliterative usefulness.

It seems highly unlikely that an enzyme of this sort preexists in the cells. All recognized cellular RNA components, including the message fraction (8), the two ribosomal components (4, 5), and the translational 4sRNA (6, 9), have been shown to be complementary to some sequences in the homologous DNA. Furthermore, actinomycin D which inhibits the DNA-dependent–RNA polymerase (10),[1] prevents synthesis of RNA in both bacterial (11) and in animal cells but does not inhibit the production of RNA viruses (12).

[1] Again, for reasons of alliterative brevity, we will hereafter refer to this enzyme as "transcriptase."

C. VIRAL RNA AS A TRANSLATABLE MESSAGE

On the basis of the arguments detailed above, we assumed that there was no mechanism in uninfected cells for using RNA to generate either complements or identical replicas. Although the transcriptase can, under certain circumstances, employ RNA as a template to synthesize complementary fragments, it does so very poorly. It was our feeling from the outset that this reaction would not normally be employed as a step in virus replication. Consequently, when we began our enzymological investigations, much of our efforts were directed at eliminating transcriptase from our preparations. Complete justification for this view was finally provided and is described in subsequent sections dealing with the properties of purified replicases. In any event, this line of reasoning did lead to the prediction that the entering RNA must itself serve as a protein program and be directly translatable in order to communicate with the cell. In this respect, it is quite unlike the single-stranded DNA virus, ϕX-174. In this case the mature virus particle contains the nonsense or noncoding strand (13) which, on being injected into the host, is converted into a double-stranded structure by a preexisting enzyme in the host cell. No such device is available to the RNA viruses.

Direct proof that viral RNA can serve as a protein program was achieved in an in vitro system by Nathans et al. (14) with f2 and confirmed by Ohtaka and Spiegelman (15) with MS-2. Viral RNA is capable of inducing the synthesis of a protein which chromatograms with the coat protein and possesses a peptide fingerprint pattern which coincides with it.

There is another consequence of this line of reasoning which can be and was subjected to test. Since the new kind of replicase must be synthesized *before replication can begin*, it follows that the entering RNA *must be conserved* while serving as a protein program. Without conservation there would be nothing left to replicate by the time the replicase was completed. Doi and Spiegelman (16) undertook to test the validity of this prediction by the use of MS-2 in which the RNA was doubly labeled with ^{15}N and ^{32}P. The RNA recovered after completion of lysis was banded in gradients of cesium sulfate. The experiments were carried out under conditions which avoided the ambiguity which would be generated by the presence of nonparticipating strands or inactive virus particles. The two isotopes were recovered in the same RNA strands corresponding in density to the ^{15}N-^{32}P–RNA originally injected.

The data obtained are consistent with the conclusion that the parental strands of an RNA virus are completely conserved during all the replications and translations required to produce a full yield of mature virus particles. Parenthetically it may be noted that these results demonstrate that, even in *E. coli*, instability is not a mandatory attribute of RNA molecules which serve as programs for protein synthesis.

D. POSSIBLE A PRIORI MECHANISMS OF RNA REPLICATION

Before we attempt to detail the more recent enzymological approaches to the problem of RNA replication, it is of interest to review briefly the various possibilities that can be entertained about the nature of RNA replication as derived from arguments of varying plausibility. The kinds of imaginable mechanisms can be divided into two classes. One presumes that Nature is pleasantly uniform and that the nucleic acid universe is completely describable by "Watson-Crickery." One predicts as a consequence that the replication of single-stranded RNA will mimic the mechanism employed by its counterpart, single-stranded DNA. The alternative view considers the possibility that RNA genomes evolved a uniquely different duplicating device. We may now consider the a priori reasons which can be marshalled in favor of each line of thought.

1. *The Unique Solutions to RNA Replication*

In the first place, one can argue quite plausibly that since the RNA viruses are mandatory intracellular parasites, they must have arisen after cells evolved. Consequently, the RNA viruses emerged in a complicated biochemical environment containing highly complex enzyme molecules fashioned to carry out reactions demanding refined levels of selective specificity. These were available to the RNA viruses for choice and modification to suit their particular needs. In contrast, DNA organisms emerged in a comparatively primitive biochemical environment. They had to solve the problem of replication without the aid of the more recently evolved sophisticated protein catalysts. Of necessity, DNA was initially forced to employ its hydrogen-bonding capacities and to use the principle of complementarity to duplicate. The *apparent* use of this mechanism today may simply represent a residue of the difficulties which DNA encountered early in its evolution. We emphasize the qualifications "apparent" since despite a widespread belief to the contrary, the fact is that *we do not know how DNA duplicates in detail*. No one has as yet produced incontrovertible evidence which compels us to accept the conclusion that base pairing on the template is a step which necessarily precedes the addition of the next complementary residue to the growing chain. One can still entertain a mechanism in which the enzyme makes the choice via "allosteric instruction" from the template; base pairing would then occur subsequent to the synthesis of the new diester bond. Indeed evidence is beginning to appear (17) indicating that the DNA polymerase is playing a key role in selecting the triphosphate to be added to the growing chain on the complementary template.

There is another argument one can offer in favor of a unique solution for

RNA replication. We have seen that RNA genomes are already translatable messages. In view of what we know about the coding dictionary (e.g., UUU is not equal to AAA) the complement of a translatable message is likely to be nonsense. Consequently, complementary transcription is not only unnecessary for transmission of information, but is indeed likely to result in the formation of a strand useless for protein synthesis. Thus, if complementary copying does occur as a step in the process of RNA replication, it would be employed only for replicative purposes. Synthesis of polynucleotide strands is energetically very expensive, and avoiding this step would provide an obvious advantage to RNA viruses. In any event, because of these and other considerations, we pointed out (16) that RNA viruses may yet furnish us with a surprising variation on the Watson-Crick theme.

2. *DNA-Like Solutions for RNA Replication*

The most popular and widely adopted model stems from the studies of the single-stranded (19) DNA virus ϕX-174, the relevant properties of which may be briefly summarized. On infection, the single-stranded DNA is converted into a double-stranded structure which has been named (20) the "replicative" form (RF-DNA). It can be shown (21) by column chromatography and prelabeled virus that this conversion is complete even at elevated multiplicities of infection.

It is presumed that the RF-DNA then serves as a template for the formation of single-stranded copies via an asymmetric synthesis analogous to the mode of action of the transcriptase. Here it should be noted that this mechanism of replication has not as yet been established. Proof that the RF-DNA is a replicative form remains to be provided. Hayashi and Spiegelman (13, 22) showed that the single strand found in the mature virus particle is the nonsense strand. It follows that in order for this virus to communicate with its host, the complement must be synthesized. Consequently, finding a double-stranded structure does not necessarily signify that the replicative form has been identified. Its presence is already justified by its requirement for transcription. Indeed, even if a single-stranded DNA virus were discovered which carried the coding strand, we would still predict the intervention of a duplex. The cellular transcription mechanism which viruses must use is designed to make single-stranded copies of RNA from a double-stranded template, so that in any event, the duplex would have to be completed.

The point of all this is that the DNA duplicating model which served as a departure point for devising the RNA replicating model is itself not certain. Indeed the recent experiments of Denhardt and Sinsheimer (23, 24) demonstrate that not all the RF-DNA molecules found in the infected complex are used as replicating templates.

Despite these reservations, one must grant the attractiveness of assuming

that a single-stranded RNA virus would have the same general problems as a single-stranded piece of DNA and that RNA might well therefore adopt the same pathway for its molecular life history. Ochoa et al., therefore proposed (18) that the first step in the replication of an RNA would be the conversion of the incoming RNA into a double-stranded structure which could then serve as a replicative form for the generation of single-stranded copies of the mature viral RNA. According to this view, replication of RNA viruses introduced no novelties. The same general rules, assumed to function in the ϕX-174 system, were presumed to apply here also.

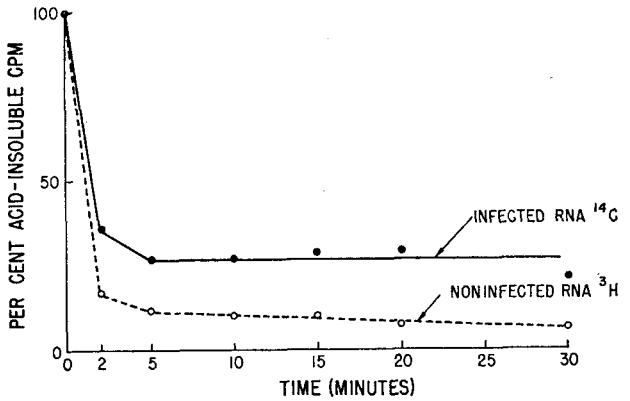

FIGURE 2. Ribonuclease resistance of bulk RNA from MS-2–infected (^{14}C) and noninfected (^{3}H) cells. RNAase treatment, 3 ml sample of a mixture of ^{3}H-RNA (60,000 CPM) and ^{14}C-RNA (40,000 CPM) in 0.01 M Tris + 5×10^{-3} M $MgCl_2$ + 0.2 M NaCl incubated at 30°C with 30 μg RNAase. 0.1 ml aliquots removed at intervals and acid-insoluble radioactivity counted on membrane filters in a liquid scintillation spectrometer (Overby, et al., unpublished observations).

E. THE SEARCH FOR A DOUBLE-STRANDED RNA

Not only was the idea of a duplex replicating form attractive, but also what appeared to be supporting evidence quickly accumulated in the literature on the RNA bacteriophages (25–31). Structures were found in infected cells having some of the properties of a double-stranded RNA. We may briefly illustrate the nature of the evidence with results obtained some time ago in our own laboratory and which are in agreement with many of the reports just cited.

The following properties can be used in the search for evidence of double-stranded RNA. (*a*) Comparative resistance to RNAse; (*b*) a lower density in a Cs_2SO_4 gradient; and (*c*) conversion to RNAse sensitivity by heating and fast cooling at low ionic strength.

It is comparatively easy to demonstrate the existence of RNA components in infected cells which do indeed possess some of the expected features. Fig. 2

describes a fairly simple experiment illustrating the RNAse resistant property. Bulk RNA is prepared from cells labeled with ^{14}C-uridine during infection with bacteriophage MS-2 and a control ^{3}H-labeled preparation is isolated from noninfected cells. Aliquots of the two RNA preparations are mixed and subjected simultaneously to ribonuclease digestion. It is evident from Fig. 2 that there is a component present in the RNA derived from infected cells

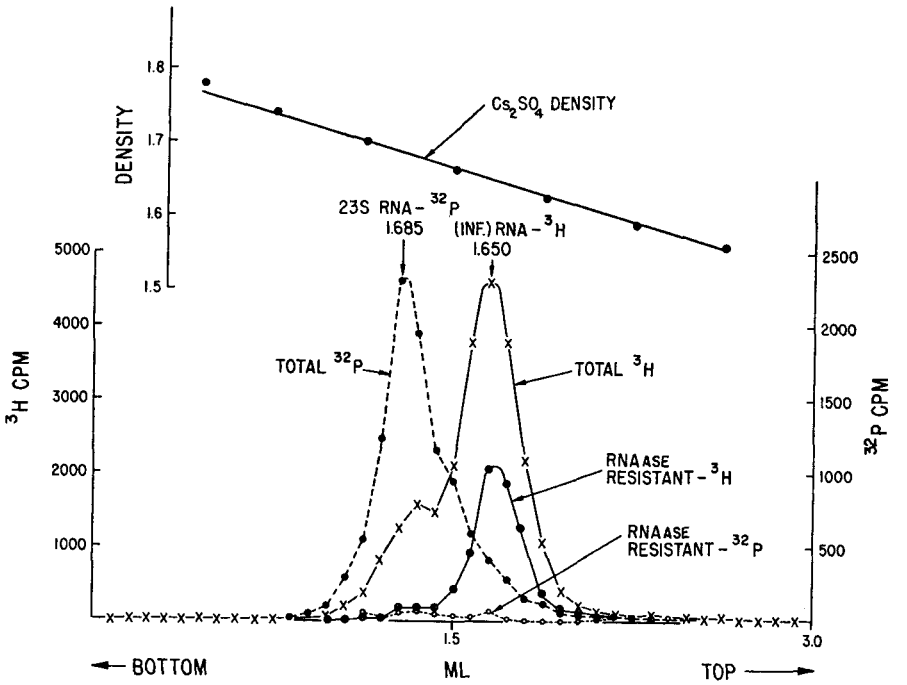

FIGURE 3. Cesium sulfate density gradient centrifugation of ^{3}H-RNA from MS-2–infected E. coli. The sample contained 35 μg ^{3}H-RNA (20,000 CPM) extracted from an infected culture, labeled with ^{3}H-uridine 40 to 45 min after infection, plus a marker of *E. coli* 23S RNA-^{32}P. Aliquots of collected samples were counted for acid-insoluble radioactivity on membrane filters in a liquid scintillation spectrometer before and after RNAase treatment. The aliquot was diluted 60 to 1 with 0.01 M Tris + 1×10^{-3} M $MgCl_2$ and incubated at 30°C for 20 min with 10 μg RNAase per ml (Overby et al., unpublished observations).

which is more resistant to ribonuclease than the corresponding noninfected control.

To examine the density of the resistant structure, RNA was prepared from cells labeled with ^{3}H-uridine late in infection (40 to 45 min) to minimize incorporation into normal components and ^{32}P ribosomal RNA from uninfected cells was added as a marker. Fig. 3 shows the profile obtained in a Cs_2SO_4 gradient. Most of the ^{3}H has entered into a well-defined peak distinct from the ribosomal and occupying a density position characteristic of viral RNA. Exam-

ination of RNAse resistance along the gradient shows the usual low core in the ^{32}P-RNA and a peak of resistant ^{3}H-RNA at a mean density slightly lighter than that of the single-stranded mature viral RNA. Finally, the last expected feature is shown in Fig. 4. The RNAse resistance structure is converted into one which is sensitive to RNAse by heating to 95°C and fast cooling.

These results confirm the experiments of many other laboratories using a variety of viruses. Everything looks reasonable and consistent with the interpretation involving a double-stranded intermediate in RNA replication. However, closer examination reveals certain difficulties in accepting this as compelling evidence that we have unveiled a component in the replicative pathway of viral RNA. The first disturbing feature is the fact that only a very small percentage of the injected viral RNA strands is to be found in this structure. In this respect, it is completely different from the case of ϕX-174, in which every injected strand is converted into double-stranded RF-DNA. Second, one unfortunately finds that this material tends to accumulate late in infection, long after many mature single strands have been made. This is not easily reconcilable with the resistant structure being a mandatory initial intermediate. Again, this is in striking contrast to the ϕX-174 situation where the first thing to be observed is the accumulation of RF-DNA long before the appearance of single-stranded mature DNA. Finally, a detailed examination of

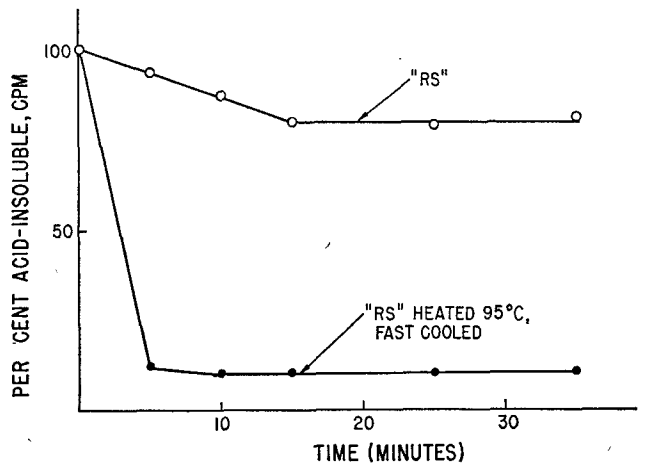

FIGURE 4. Effect of heating on RNAase sensitivity of RS-RNA-^{3}H. Heating procedure sample (20,000 CPM) in 6.4 ml of 0.01 M Tris + 10^{-4} M EDTA + 0.015 M NaCl divided into two equal portions. One was heated from 25° to 95°C at a rate of about 1°/min and then fast cooled in ice water. RNAase digestion, heated and unheated samples were made 0.15 M in NaCl, and incubated at 30°C with 10 μg/ml of RNAase. 0.5 ml aliquots were removed at intervals and acid-insoluble radioactivity determined (Overby et al., unpublished observations).

the properties of the so-called RF-RNA is not consistent with a 1:1 duplex of parent and complementary strand. Again, this is not analogous to the situation found with ϕX-174 in which the second strand made on the first is a complete complement.

One can argue that since resistant structures are found only in infected cells, it must have some relevance to replication. While plausible, such arguments do not have the ring of logical necessity. One can grant that the resistant structure is a *consequence* of the infective process without accepting it as a mandatory component of the replicative process. It may play some other undetermined role or be a nonfunctional artifact. One must bear in mind that we are dealing here with infected cells which are well along the path to death, and it is not outside the realm of possibility that pathological artifacts might be produced. Under these circumstances, more direct evidence than mere existence must be provided before the resistant structures are accepted as demonstrated components of the replicative process.

In any case, our own attempts and those of others to study the process in the infected cell convinced us that it would be difficult to design a truly decisive experiment which could hope to settle the question of the role of these RNAse-resistant structures and their relation to RNA replication. It seemed necessary to get on with the enzymology in the hope that the relevant enzyme system could be purified to the point at which the mechanism of RNA synthesis could be examined in a simple system containing only known components.

F. THE SEARCH FOR THE MS-2 REPLICASE

The search for a unique RNA-dependent polymerase is complicated by the presence of a variety of enzymes which can incorporate ribonucleotides either terminally or subterminally into preexistent RNA chains (32). In addition, there are others (e.g., RNA phosphorylase (33), polyadenylate synthetase (34), etc.) which can mediate extensive synthesis of polynucleotide chains. Many of these sources of confusion can be avoided by suitable adjustment of the assay conditions and supplementary tests for requirement of all four ribosidetriphosphates. The most serious difficulty is introduced by the DNA transcriptase since it can employ, though poorly, certain types of RNA as substitute templates for RNA synthesis (35, 36). Under these circumstances the use of actinomycin D or DNAase does not insure against observing its activity. The only certain way to avoid interference from the DNA-dependent enzyme is to eliminate it from the fraction of interest.

The considerations noted above make it obvious that a claim for a new type of RNA polymerase must be accompanied by evidence for RNA dependence and a demonstration that the enzyme possesses some unique characteristic which differentiates it in one or more of its properties from previously known enzymes with which it can be confused.

In addition to these enzymological difficulties, we recognized a biological feature of the situation which influenced in at least one important detail the procedure we chose in the search for replicase. The point at issue may perhaps best be described in rather naive and admittedly somewhat anthropomorphic terms. Consider an RNA virus approaching a cell some 10^6 times its size and into which the virus is going to inject its only strand of genetic information. Even if the protein-coated ribosomal RNA molecules are ignored, the cell cytoplasm still contains of the order of 10,000 free RNA molecules of various sorts. The viral RNA contains the information required for the synthesis of the new kind of polymerase designed to make RNA copies from RNA. If this replicase were indifferent and accepted any RNA it happened to meet, *what chance would the single original strand injected have of ever being replicated?*

Admittedly there are several ways out of this dilemma. One could, for example, segregate the new polymerase molecule and the viral RNA in some sequestered corner where they would be isolated and undisturbed by the mass of cellular RNA components. However, the unique possibility we entertained is that the virus is ingenious enough to design a polymerase which would recognize its own genome and ignore all other RNA molecules.

At the outset, of course, we did not know which solution had been adopted by the virus to solve this dilemma, or even if the dilemma were real. However, the possibility that it did exist and that replicase selectivity might be the chosen solution required that its implications not be ignored; for if true, its disregard would guarantee failure. In particular, it meant that we could not afford the luxury of using any conveniently available RNA. This view demanded the use of purified viral RNA in all steps of the search for replicase. Further, one might perhaps push the selectivity property to its ultimate pessimistic conclusion. If the cleverness of the replicase extends further, it might well be true that even a fragment of its own genome would not be recognized and accepted for replication. This added possibility made it necessary to provide a guarantee that the RNA employed is not only homologous, but also intact. This in turn introduced the complication that stages of purification preceding the removal of ribonuclease could well yield ambiguous or even false clues even with intact homologous RNA. Thus, one had to "fly blind" in the initial steps and depend on very brief assays to provide the guides for the direction of the subsequent stages.

Despite all these potential obstacles, many of which were actually realized, the first success was achieved (37). A procedure was finally devised involving negative protamine fractionation and column chromatography which yielded what looked like the relevant enzyme from *E. coli* infected with MS-2. The enzyme required all four triphosphates and on the basis of a nearest neighbor analysis, it was clearly synthesizing a proper heteropolymer. Most important of all, the preparation exhibited a virtually complete dependence on added

RNA. This provided the opportunity for subjecting the expectation of specific template requirement to experimental test. The response of MS-2 replicase to various kinds of nucleic acid is given in Table I. We note immediately that there indeed is a striking preference for its own RNA. No significant activity is observed with either the host sRNA or ribosomal RNA. Further, ribosomal RNA is unable to interfere detectably with the template activity of the MS-2

TABLE I

TEMPLATE SPECIFICITY OF PURIFIED RNA-DEPENDENT POLYMERASE

(Haruna et al., 37)

The standard reaction volume of 0.25 ml contained the following in μM: Tris-HCl pH 7.5, 21; $MgCl_2$, 1.4; $MnCl_2$, 1.0; KCl, 3.75; mercaptoethanol, 0.65; spermine, 2.5; phosphoenolpyruvate (PEP), 1.0; $(NH_4)_2SO_4$, 70; CTP, ATP, GTP, and UTP, 0.5 each. In addition, it contained pyruvate kinase (PEP kinase), 5 μg; DNAase, 2.5 μg; and where indicated, 10 μg of the polynucleotide being tested as template. Enzyme was assayed at levels between 50 to 300 μg protein per sample. DNAase was always omitted in assaying for DNA-dependent polymerase activity. Incubations were carried out at 35°C for 10 min and terminated by placing the reaction mixture in an ice bath and by the addition of 0.15 ml of neutralized saturated pyrophosphate, 0.15 ml of neutralized orthophosphate, and 0.1 ml of 80% trichloroacetic acid (TCA). The precipitate was washed onto a Millipore filter and washed five times with 10 ml of cold 10% TCA containing 0.9% of Na pyrophosphate. The Millipore membrane was then dried and counted in a liquid scintillation counter as described previously (8). The pyrophosphate is included in the wash to depress the zero time backgrounds to acceptable levels (40 to 70 CPM per sample containing input counts of 1×10^6 CPM).

Template all at 10 μg/0.25 ml	NT incorporated in mμmoles/10 min/mg protein
0	0.08
MSϕ2-RNA	8.5
sRNA	0.09
Ribosomal RNA	0.06
Ribosomal RNA + MSϕ2 RNA	8.0
TMV RNA	0.3
TYMV RNA	2.2
CT-DNA*	0.11

* DNAase omitted from assay mixture.

RNA. Of the two heterologous viral RNA's tested, tobacco mosaic virus (TMV) exhibits a low activity and turnip yellow mosaic virus (TYMV) is somewhat better. However, as will be seen below, this latter activity is severely repressed if Mn^{++} is omitted from the reaction mixture. We shall return to this point later.

The negligible response to calf thymus DNA (CT-DNA) shows clearly that the DNA-dependent RNA polymerase is absent from our enzyme preparation and the properties exhibited cannot therefore be ascribed to contaminating

transcriptase. Further, as a final check, purified transcriptase possessing a specific activity of 6000 Chamberlin-Berg units per mg was prepared (53) from *E. coli*. When presented with the MS-2 RNA as a template it incorporated labeled ribosidetriphosphate at less than 3% of the rate of the replicase under equivalent assay conditions.

The outstanding feature of this enzyme is its clear preference for its homologous RNA as template and the virtually complete inactivity of the host ribosomal RNA and "4S" RNA. It is evident that the ability of the replicase to discriminate between one RNA molecule and another does indeed solve the crucial problem for an RNA virus attempting to direct its own duplication in an environment replete with other RNA molecules. By producing a polymerase which ignores the mass of preexistent cellular RNA, a guarantee is provided that replication is focused on the single strand of incoming viral RNA, the ultimate origin of progeny.

It seems worth noting that sequence recognition by the enzyme can be of value not only to the virus, but also to the investigator. The search for viral RNA replicases, as we have indicated, must perforce be carried out in the midst of a variety of highly active cellular polymerases capable of synthesizing polyribonucleotides. If the enzyme finally isolated possesses the appropriate template requirement, a comforting assurance is furnished that the effort expended and the information obtained are indeed relevant to an understanding of viral replication.

G. CONFIRMATION OF SPECIFIC TEMPLATE REQUIREMENTS OF RNA REPLICASES

Our line of reasoning would lead to the expectation that RNA replicases induced by other RNA viruses would show a similar preference for their homologous templates. This was, however, not a foregone conclusion since it was conceivable that other viruses might evolve different solutions to the problem of preferential synthesis. It seemed important to us to see whether this property could be observed in another virus unrelated to MS-2. At this point in our investigations, we were faced with the technical dilemma that all of the known RNA bacteriophages were serologically related and had a similar RNA base composition. It seemed unlikely that any one of them would be a suitable candidate for a test of this kind. We were forced to consider the rather unpleasant possibility of abandoning *E. coli* and turning our attention to either a plant or an animal system, with all its attendant logistic and technical difficulties.

Just at the right time Professor Watanabe from Tokyo visited our laboratory and told us about a number of RNA phages his group had isolated (38) in Japan. One of them (denoted as Qβ) sounded exciting because it looked as if it might well be different from all the others. Examination of a sample he

kindly provided confirmed Watanabe's characterization of Qβ and demonstrated that it was completely unrelated serologically to MS-2 (39). In addition (40) its coat protein was clearly different since it lacks not only the histidine missing in MS-2 protein, but methionine and tryptophan as well. Further, the base composition of Qβ RNA is unique possessing an 8% difference between the mole ratios of A and U compared to the essentially statistical tetranucleotide base composition of MS-2 and the other known RNA bacteriophages.

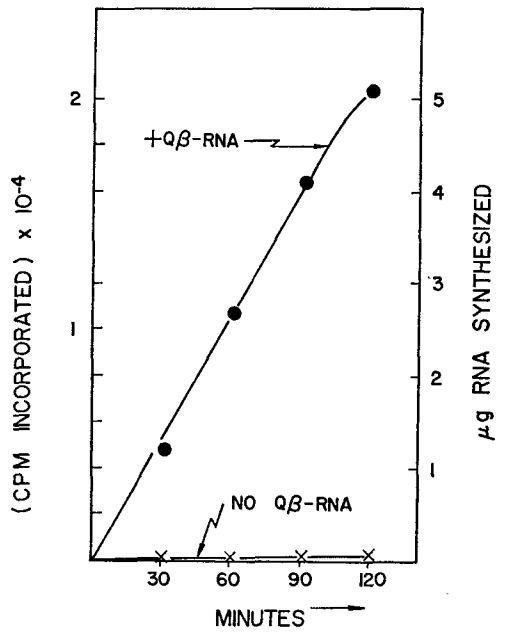

FIGURE 5. Kinetics of replicase activity. Each 0.25 ml contained 40 μg of protein and 1 μg of Qβ-RNA. See Fig. 7 for further details. The specific activity of the ^{32}UTP was such that the incorporation of 4,000 CPM corresponds to the synthesis of 1 μg of RNA (Haruna and Spiegelman, 41).

Just about the same time another helpful event occurred. As a result of an heroic undertaking in the laboratory of Watson and Gilbert at Harvard, an *E. coli* mutant (Q-13) was isolated by Vargo which lacked ribonuclease 1 and any evidence of RNA phosphorylase activity. The latter enzyme had been particularly difficult to eliminate from our preparations and the RNAase I introduced additional complications in the early stages of fractionation. Consequently, Q-13 was adopted as the host of choice in all our subsequent investigations.

The isolation and purification of the Qβ replicase (41) essentially followed, with slight modifications, the procedures worked out earlier (37) for the MS-2 replicase. The properties of the Qβ replicase on purification to the stage of complete RNA dependence, exhibited the same general features as had been observed with MS-2 replicase. These included requirement for all four triphosphates, and Mg^{++}. Mn^{++} substitutes partially, supporting only about 10%

of the synthetic rate attainable with Mg^{++}. However, Mn^{++} induces interesting abnormal changes in the nature of the reaction to which we will return below.

Fig. 5 shows the kinetics observed in a reaction mixture containing saturating amounts of template (1 μg of RNA for 40 μg protein). It should be noted that continued synthesis is observed at 35°C for periods exceeding 5 hr and that in 2 hr, the amount of RNA synthesized corresponds to five times the input of template. By variation in the amount of RNA added and the time permitted for synthesis, virtually any desired level of increase over the starting material can be achieved.

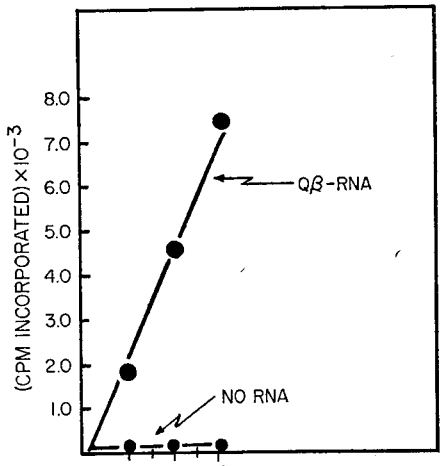

FIGURE 6. Response to added protein. Assays were run for 20 min at 35°C as in Fig. 7. Again, the incorporation of 4,000 CPM corresponds to the synthesis of 1 μg of RNA (Haruna and Spiegelman, 41).

The cessation of synthesis within 5 to 10 min reported by others for presumably similar preparations has been observed by us only in the early stages of purification prior to the removal of the nucleases. Fig. 6 examines the effect of added amounts of protein at a fixed level of template from which it is evident that the reaction responds linearly, indicating the absence of interfering contaminants in the purified enzyme.

We now turn our attention to the primary question which we sought to resolve. Table II records the abilities of various RNA molecules to stimulate the Qβ replicase to synthetic activity at saturation concentrations of homologous RNA, and twice this level. The response of the Qβ replicase is in accord with that reported for the MS-2 replicase, the preference being clearly for its own template. The only heterologous RNA showing detectable activity is TYMV and, at the 2 μg level, supports a synthesis corresponding to 6% of that observed with homologous Qβ RNA. Both the heterologous viral RNA's, MS-2 and STNV, are completely inactive, and again, so are the ribosomal and transfer RNA species of the host cell. As might be expected, bulk RNA from infected cells shows some templating function, an activity which increases as the infection is allowed to progress. It is important to note again from the absence of response to DNA that our purification procedure eliminates detectable evidence of transcriptase from our enzyme preparations.

TABLE II

RESPONSE OF Qβ REPLICASE TO DIFFERENT TEMPLATES

(Haruna and Spiegelman, 41)

Conditions of assay are those specified in Fig. 7. However, as in all cases, assay for DNA-dependent activity is carried out at 10 μg of DNA per 0.25 ml of reaction mixture. Control reactions containing no template yielded an average of 30 CPM.

Template	Input levels of RNA	
	1 μg	2 μg
Qβ	4,929	4,945
TYMV	146	312
MS-2	35	26
Ribosomal RNA	45	9
sRNA	15	57
Bulk RNA from infected cells	146	263
Satellite virus	61	51
DNA (10μg) 36		

The experiment with the RNA from the satellite virus of tobacco necrosis virus (STNV) was a particularly interesting challenge. Reichmann showed (59) that the satellite virus contains only enough RNA to code for its coat protein, from which we would infer that this virus must employ the replicase of the companion tobacco necrosis virus (TNV) for its multiplication. This implies, either that the satellite is related in sequence to the TNV virus, or that it possesses some general recognition feature permitting it to employ any viral RNA replicase. The fact that STNV-RNA does not serve as a template for

TABLE III

TEMPLATE SPECIFICITY OF TWO RNA REPLICASES

(Haruna and Spiegelman, 41)

Conditions of assay are those specified in Table II.

Enzyme	RNA templates			
	MS-2 RNA		Qβ-RNA	
	1 μg	2 μg	1 μg	2 μg
MS-2	4,744	4,366	0	56
Qβ	36	65	2,871	3,731

either one of the two purified replicases implies that the answer will be found in at least partial sequence homology between STNV and TNV genomes, a prediction open to experimental test.

To permit a definitive comparison of the two, replicases were isolated from appropriately infected Q-13. The results of the comparison are shown in Table III and they are satisfyingly clear-cut. The MS-2 replicase shows no evidence of accepting the Qβ RNA as a template at either level of RNA input. Similarly, the Qβ replicase completely ignores the MS-2 RNA while functioning quite well with its own template. It would appear from these data that the prediction of template specificity is completely confirmed for at least two viruses.

H. COMPARISON WITH OTHER PREPARATIONS DERIVED FROM CELLS INFECTED WITH RNA PHAGES

Other laboratories have reported on presumably similar enzymes from *E. coli* infected with a variety of RNA phages. The very different states of purity make it difficult to compare the properties of the replicases reported with enzymes detected (42) or isolated by others (27, 43–46). Thus, activity independent of added RNA (27, 43) suggests that the purification has not achieved an adequate removal of contaminating RNA, and, in any event, precludes an examination of template specificity. August et al. (44–46) prepared an enzyme from *E. coli* infected with SU-11 mutant of f2 which is apparently stimulated by a variety of RNA species, including host ribosomal and sRNA. It is conceivable that the f2 replicase is nonspecific in its template requirements. However, it should be noted that the August preparation is stimulated only if 20 μg of RNA is added for each microgram of protein. The inordinately large amounts of RNA required may be a reflection of the detectable contamination of their preparation with ribonuclease and the consequent need for scavenger RNA to protect the product synthesized. Under the circumstances, judgment had better be withheld on the significance of the apparent nonspecificity since it is open as yet to a rather trivial explanation.

The following features distinguish the purified replicases described here from the presumably similar preparations reported by other laboratories: (*a*) complete dependence on added RNA, (*b*) competence for prolonged (more than 5 hr) synthesis of RNA, (*c*) ability to synthesize many times the input template, (*d*) saturation at low levels of RNA, and (*e*) virtually exclusive requirement for homologous template under optimal ionic conditions.

It should be evident from the properties listed that the replicases were indeed approaching a state of purity where it became relevant to examine the nature of the product in greater detail; a necessary prelude to experiments designed to illuminate mechanism.

I. AUTOCATALYTIC SYNTHESIS OF A VIRAL RNA

The experimental analysis of a replicating reaction centers necessarily on the nature of the product. If, in particular, the concern is with the synthesis of a viral nucleic acid, data on base composition and nearest neighbors, while of interest, are hardly decisive. The ultimate issue is whether or not replicas are in fact being produced. To answer this question, information on the sequence of the synthesized RNA is required. Affirmative evidence of similarity between template and product would provide assurance that the reaction being studied is indeed relevant to an understanding of the replicative process.

The ability of a replicase to distinguish one RNA sequence from another can be used to provide information pertinent to the similarity question. Two types of readily performed experiments can decide whether the product is recognized by the enzyme as a template. One approach is to examine the kinetics of RNA synthesis at template concentrations which start below those required to saturate the enzyme. If the product can serve as a template, a period of autocatalytic increase of RNA should be observed. Exponential kinetics should continue until the product saturates the enzyme after which synthesis should become linear.

A second type of experiment is a direct test of the ability of the synthesized product to function as initiating template. Here a synthesis of sufficient extent is carried out to insure that the initial input of RNA becomes a quantitatively minor component of the end product. The synthetic RNA can then be purified and examined for its template functioning capacities, a property readily examined by means of a saturation curve. If the response of the enzyme to variation and concentration of product is the same as that observed with the viral RNA, one would have to conclude that the product generated in the reaction is as effective a template for the replicase as is RNA from the mature virus particle.

The successful performance of these types of experiments imposes rather severe demands on the purity of the enzyme preparation employed. Clearly, it must be free from interfering and confounding activities so that the reaction can be studied in a simple mixture containing, in addition to enzyme, only the required ions, substrates, and templates. We now briefly describe experiments of this nature (47) carried out with purified Qβ replicase functioning on intact Qβ RNA.

To examine for the occurrence of autocatalytic kinetics the saturation concentration of template must first be established. The response of the purified replicase to increasing concentrations of Qβ RNA is given in Fig. 7 which shows that 40 μg of enzyme is saturated by approximately 1 μg of template RNA. An experiment was therefore set up in which the ratio of input template

to protein was one-fifth of the saturation value. The results are plotted in Fig. 8 arithmetically and semilogarithmically against time to permit ready comparison of kinetics. Exponential increase of RNA is evident over a period of approximately 3 hr. The arrows indicate the time at which the kinetics depart from the exponential and become linear. Extrapolation to the ordinate indicates that the change to linear synthesis occurs when approximately 1 μg of RNA has accumulated. It will be noted that by the time the experiment was

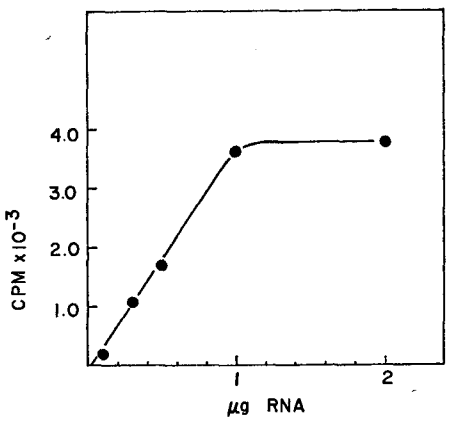

FIGURE 7. Template saturation of replicase. In addition to 40 μg of enzyme protein, each standard reaction volume of 0.25 ml contained the following in micromoles: Tris HCl, pH 7.4, 21; $MgCl_2$, 3.2; CTP, ATP, UTP, and GTP, 0.2 each. The reaction is run for 20 min at 35°C and terminated in an ice bath by the addition of 0.15 ml of neutralized saturated pyrophosphate, 0.15 ml of neutralized saturated orthophosphate, and 0.1 ml of 80% trichloroacetic acid. The precipitate is transferred to a membrane filter and washed 7 times with 5 ml of cold 10% TCA. The membrane is then dried and counted in a liquid scintillation spectrometer. The washing procedure yields zero time values of 80 CPM with input counts of 1×10^6 CPM. The radioactively labeled ^{32}UTP was synthesized as detailed earlier (37) and was used at a level of 1×10^6 CPM/0.2 μmole. The enzyme was isolated from *E. coli* (Q13) infected with bacteriophage Qβ as detailed by Haruna and Spiegelman (41, 47).

terminated, the RNA synthesized corresponded to approximately twenty-five times the initial seed.

The results just described are consistent with the implication that the product produced in the course of the reaction can serve to stimulate new enzyme molecules to activity. The enzyme is therefore able to recognize the product as being one which is homologous to its own genome.

To carry out the more direct test of this conclusion, a 1 ml reaction mixture was set up as detailed in Table IV and the synthesis allowed to proceed for 3.5 hr by which time a more than 60-fold increase of the input material was achieved. The reaction was then terminated and the RNA purified by the phenol method which yielded 55% of the synthesized product. Fig. 9 shows

the size distribution in a linear sucrose gradient of the synthesized ^{32}P product compared with Qβ RNA freshly isolated from virus. It is clear that much of the product has the 28S size characteristics of Qβ RNA. There is evidence of some smaller fragments which may represent either abortive or incomplete synthesis.

Since the product was ^{32}P-labeled it was necessary to examine its template

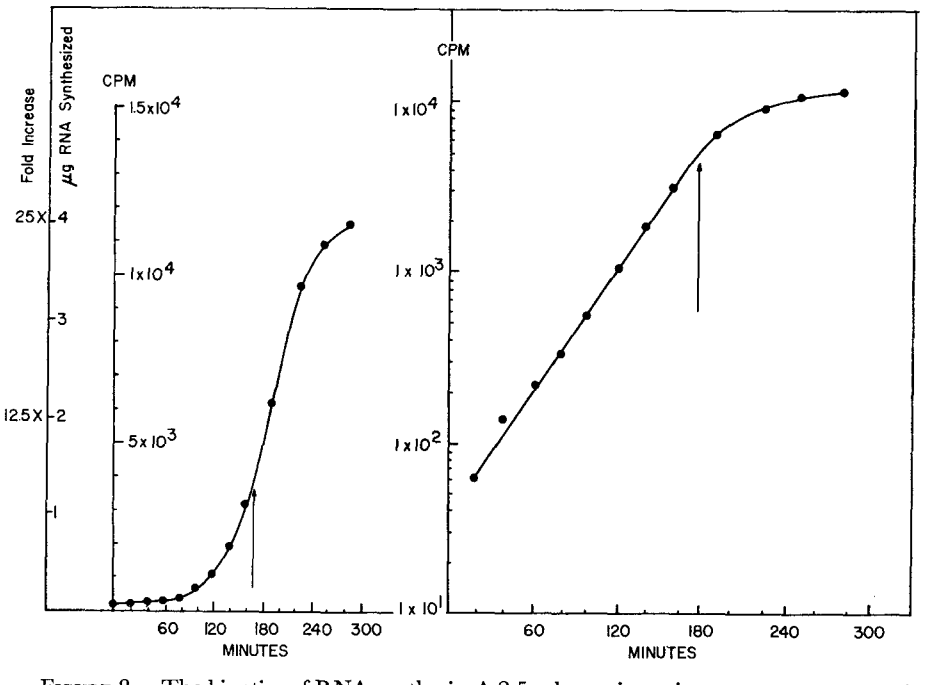

FIGURE 8. The kinetics of RNA synthesis. A 2.5 ml reaction mixture was set up containing the components at the concentrations specified in the legend for Fig. 7. The mixture contained 400 μg of enzyme protein and 2 μg of input Qβ-RNA so that the starting ratio of template to enzyme was one-fifth of the saturating level. At the indicated times, 0.19 ml aliquots were removed and assayed for radioactive RNA as detailed in Fig. 7. The ordinates for counts per minute and micrograms of RNA synthesized refer to those found in 0.19 ml samples. The data are plotted against time arithmetically on the right, and semilogarithmically on the left. The arrows indicate change from autocatalytic to linear kinetics (Haruna and Spiegelman, 47).

in function using another identifying isotope (^{3}H-UTP) and this was carried out as detailed in the legend for Fig. 10 which illustrates the response of the replicase to various input levels of the product (triangles) compared to the original viral RNA (circles). It is evident that the RNA synthesized in the course of the 60-fold increase is as effective in serving as a template as the original viral RNA.

The data we have just summarized support the assertion that the reaction generates a polynucleotide of the same molecular weight (1×10^6) as viral

RNA and which the replicase cannot distinguish from its homologous genome. It is clear that the enzyme is faithfully copying the recognition sequence employed by the replicase to distinguish one RNA molecule from another.

The next question concerns the extent of the similarity between product and

TABLE IV

A 60-FOLD SYNTHESIS OF VIRAL RNA

(Haruna and Spiegelman, 47)

A 1 ml reaction volume contained the following in micromoles: Tris HCl, pH 7.4, 84; $MgCl_2$, 12.8; CTP, ATP, UTP, and GTP, 0.8 each. To this was added 160 μg of enzyme protein and 4 μg of Qβ-RNA as template. The specific activity of the ^{32}UTP was adjusted so that 1×10^5 CPM is equivalent to 25.4 μg of RNA. The mixture was incubated at 35°C and at the indicated intervals 20 μl were removed and acid-precipitable RNA assayed as described in Fig. 7. At the end of the reaction, the RNA was purified as follows: the volume is adjusted to 2 ml with TM buffer (10^{-2} M Tris, 5×10^{-3} M $MgCl_2$, pH 7.5). Two ml of water-saturated phenol is then added and the mixture shaken in heavy wall glass centrifuge tubes (Servall, 18 x 102 mm) at 5°C for 1 hour. After separation of the water phase from the phenol by centrifugation at 11,000 RPM for 10 min, another 2 ml of TM buffer is added to the phenol which is then mixed by shaking for 15 min at 5°C. Again, the phenol and water layers are separated, and the two water layers combined. Phenol is eliminated by two ether extractions, care being taken to remove the phenol from the walls of the centrifuge tubes by completely filling them with ether after each extraction. The ether dissolved in the water phase is then removed with a stream of nitrogen. The RNA is precipitated by adding 1/10 volume of potassium acetate (2 M) and 2 volumes of cold absolute ethanol. The samples are kept for 2 hr at −20°C before being centrifuged for 1 hr at 14,000 RPM in a Servall SS 34 rotor. The pellets are drained in a vacuum desiccator for 6 to 8 hr at 5°C and the RNA is then dissolved in 1 ml of buffer (10^{-2} M Tris, 10^{-2} M $MgCl_2$, pH 7.5). TCA-precipitable radioactivity is measured on 20 μl aliquots of the final product from which the per cent recovery of synthesized RNA can be determined.

Time	CPM/20 μl	RNA synthesized	Total RNA
min		*μg/ml*	
0	80	0	4
60	4,917	63	67
150	15,641	199	203
210	20,113	256	260

template. Have identical replicas been in fact produced? The most decisive test would determine whether the product contains all the information required to program the synthesis of complete virus particles in a suitable test system. The success we have just recorded encouraged an attempt at the next phase of the investigation which would subject the synthesized RNA to this more rigorous challenge.

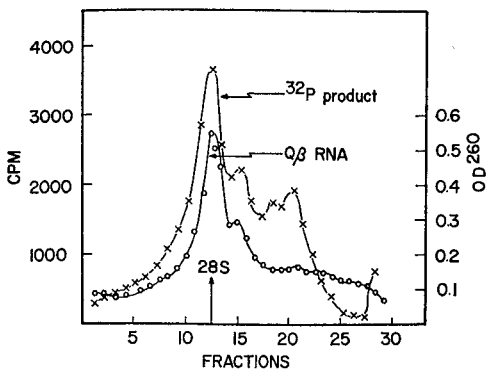

FIGURE 9. Size distribution of the synthesized RNA compared to Qβ-RNA. An aliquot of the material recovered from the 60-fold synthesis detailed in Table IV was layered on a linear (2.5 to 15%) sucrose gradient made up in 0.01 M Tris (pH 7.4) and 5×10^{-3} M Mg^{++}. It was then centrifuged for 12 hr at 25,000 RPM and 4°C in an SW 25 Spinco rotor. Samples were removed from the bottom and measurements carried out for optical density at 260 mμ and acid-precipitable radioactivity as described in the legend for Fig. 7 (Haruna and Spiegelman, 47).

J. SYNTHESIS OF AN INFECTIOUS SELF-REPLICATING VIRAL RNA

In designing experiments which involve infectivity assays of enzymatically synthesized RNA, it is important to recognize that even highly purified en-

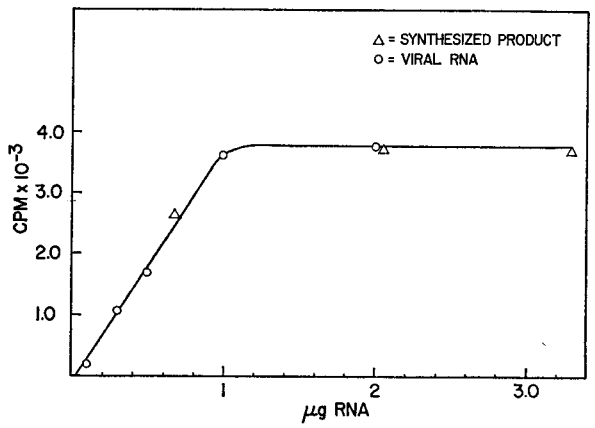

FIGURE 10. Saturation of enzyme by synthesized RNA compared to viral RNA. The experiment was carried out exactly as detailed in the legend for Fig. 7. The circles refer to the values obtained with RNA isolated from virus particles in the experiment of Fig. 7, and the triangles to the rates obtained with the RNA synthesized in the experiment of Table IV. Since in the latter case, the template used was labeled with ^{32}P, ^{3}H-UTP at 1×10^6 CPM per 0.2 μmole was used to follow the synthesis. All preparations and counting of samples were carried out as described in Fig. 7 (Haruna and Spiegelman, 47).

zymes derived from infected cells are likely to include some virus particles. Chemically, the contamination is trivial, amounting to 0.16 μg of nucleic acid and 0.8 μg of protein for each 1,000 μg of enzyme protein employed. Since 40 μg of protein are used for each reaction mixture, the contribution to the total RNA by the particles is only 0.006 μg which is to be compared with

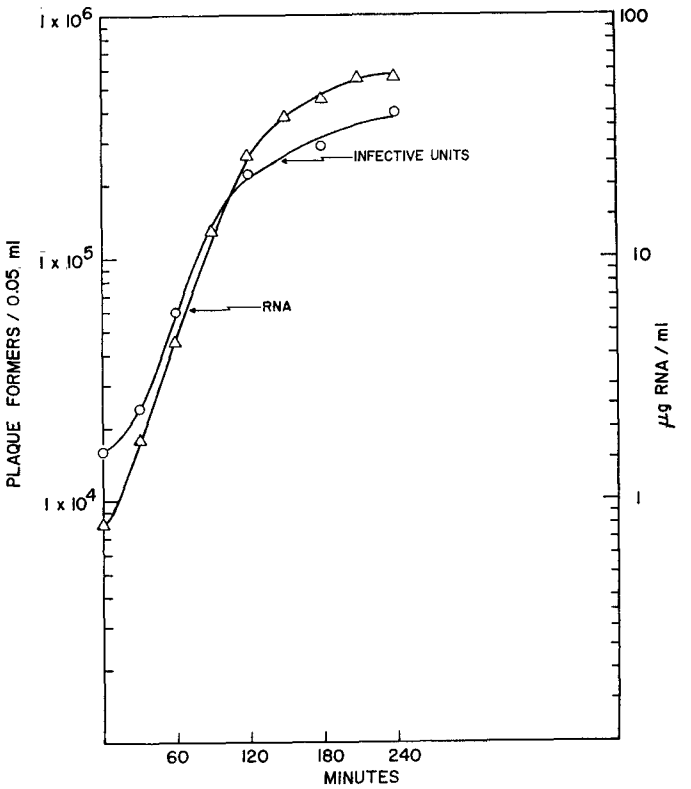

FIGURE 11. Kinetics of RNA synthesis and formation of infectious units. An 8 ml reaction mixture was set up containing the components at the concentrations specified in Fig. 7. Samples were taken as follows: 1 ml at 0 time and 30 min, 0.5 ml at 60 min, 0.3 ml at 90 min, and 0.2 ml at all subsequent times. 20 μl were removed for assay of incorporated radioactivity as described in Fig. 7. The RNA was purified from the remainder (Table IV), with radioactivity determined on the final product to monitor recovery (Spiegelman *et al.*, 48).

a 0.2 μg of input RNA and 3 to 20 μg synthesized in the usual experiment. The mandatory requirement for added RNA proves that within the incubation times used, this small amount of RNA is either inadequate or unavailable for the initiation of the reaction. Thus, these virus particles do not significantly influence either the chemical or enzymatic aspects of the experiment. However, because of their high infective efficiency compared to free RNA, even

moderate amounts of intact virus cannot be tolerated in the examinations of the synthesized RNA for infectivity. Consequently, in the experiments to be described, all RNA preparations were first phenol-treated prior to assay. Further, the phenol-purified synthetic RNA was routinely tested for whole virus particles by assay on intact cells, and none was found in the experiments reported.

1. *Comparison of the Kinetics of Appearance of RNA and Infectious Units*

We now summarize experiments (48) in which the kinetics of the appearance of new RNA and infective units were examined in two different ways. The first shows that the accumulation of radioactive RNA is accompanied by a proportionate increase in infective units. The second proves, by a serial dilution experiment, that the newly synthesized RNA is infective.

To compare the appearance of newly synthesized RNA and the presence of infectious units in an extensive synthesis, a reaction mixture was set up containing the necessary components at the concentrations required. Aliquots were taken at the times indicated for the determination of radioactive RNA and purification of the product for infectivity assay.

The results of a typical experiment are summarized in Fig. 11 in the form of a semilogarithmic plot against time of the observed increase in both RNA and infectious units. Further details are given in the corresponding legend. The amount of RNA (0.8 μg per ml at 0 time) is well below the saturation level of the enzyme present. Consequently, the RNA increases autocatalytically for about the first 90 min followed by a synthesis which is linear with time. We note in Fig. 11 that the increase in RNA is paralleled by a rise in the number of infectious units. During the 240 min of incubation, the RNA experiences a 75-fold increase and the infectious units a 35-fold increase over the amount present initially. These numbers are in agreement within the accuracy limits of the infectivity test.

Experiments carried out with other enzyme preparations yielded results in complete accord with those just described. Another example is given in Fig. 12 in which the enzyme used was pycnographically purified in a cesium chloride density gradient which decreases the virus particle content by a factor of 10^6 without change in the properties of the enzyme. An examination here reveals that again one has parallel increases in both RNA and the infectious units.

2. *Proof That the Newly Synthesized RNA Molecules Are Infectious*

The kind of experiments just described offer plausible evidence for infectivity of the newly synthesized radioactive RNA. They are not, however, conclusive since they do not eliminate the possibility that the agreement observed is fortuitous. One could argue, however implausibly, that the enzyme is "acti-

vating" the infectivity of the input RNA while synthesizing new noninfectious RNA, and that the rather complex combination of exponential and linear kinetics of the two processes happens to coincide fortuitously.

Direct proof that the newly synthesized RNA is infectious can, in principle,

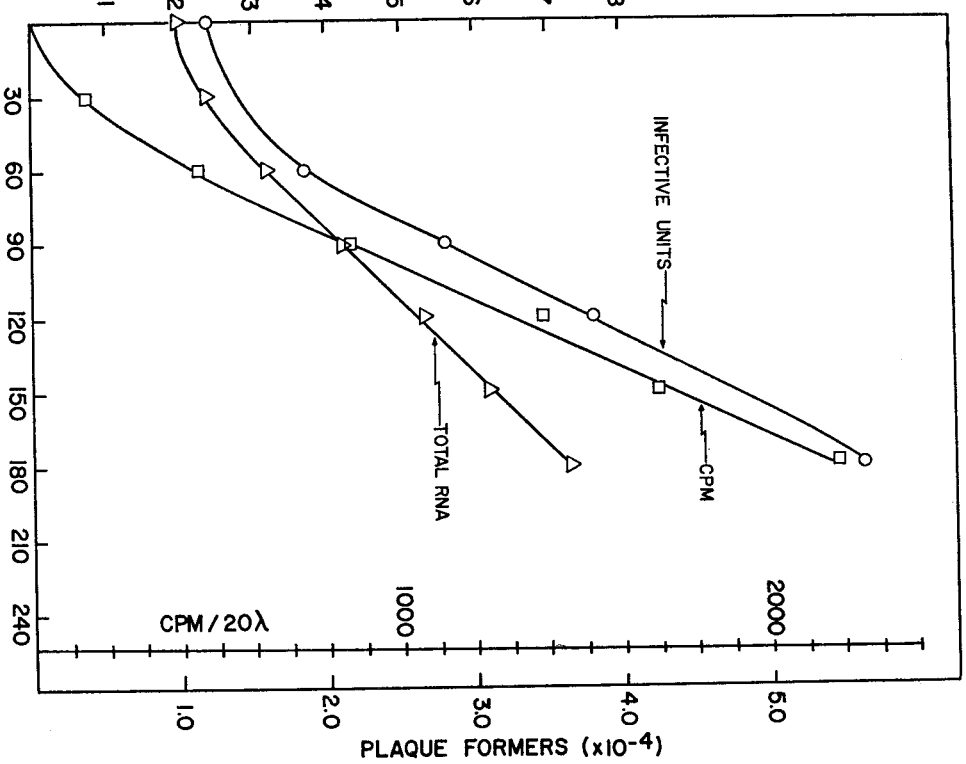

FIGURE 12. Kinetics of RNA synthesis and formation of infectious units. Same conditions as in Fig. 11 except that the enzyme was purified in a CsCl gradient which decreased the virus particle count by a factor of 1×10^6 (Spiegelman et al., unpublished observations).

be obtained by experiments which employ ^{15}N-^{3}H–labeled initial templates to generate ^{14}N-^{32}P product. The two can then, in principle, be separated in equilibrium density gradients of cesium sulfate. Such experiments have been carried out for other purposes and will be described elsewhere. However, the

TABLE V

SERIAL TRANSFER EXPERIMENT

(Spiegelman et al., 48)

Sixteen reaction mixtures of 0.25 ml were set up, each containing 40 μg of protein and the other components specified for the "standard" assay (Fig. 7). 0.2 μg of template RNA was added to tubes 0 and 1; RNA was extracted from the former immediately, and the latter was allowed to incubate for 40 min. Then 50 μl of tube 1 were transferred to tube 2, which was incubated for 40 min, and 50 μl of tube 2 then transferred to tube 3 and so on, each step after the first involving a 1 to 6 dilution of the input material. Every tube was transferred from an ice bath to the 35°C water bath a few minutes before use to permit temperature equilibration. After the transfer from a given tube, 20 μl were removed to determine the amount of ^{32}P-RNA synthesized, and the product was purified from the remainder as described in Table IV. Control tubes incubated for 60 min without the addition of the 0.2 μg of RNA showed no detectable RNA synthesis, nor any increase in the number of infectious units.

All recorded numbers are normalized to 0.25 ml. Columns 1, 2, and 3 give the transfer number, the time interval permitted for synthesis, and the elapsed time from zero respectively. Column 4 records the amount of radioactive RNA found in each tube at the end of the incubation, column 5 the total RNA in each, and 6 gives the net synthesis during the time interval. Column 7 lists the cumulative synthesis of RNA. The decreasing concentrations of the input RNA resulting from the serial dilutions are recorded in terms of micrograms (column 8), number of strands (column 9), and infectious units (IU) per tube (column 10). The last is calculated from column 9 and from an efficiency of plating (e.o.p.) of 5×10^{-7}. Column 11 lists the increment in infectious units observed during each period of synthesis, corrected for the efficiency of recovery (column 14) and column 12 represents the corresponding sum. Column 13 is the plating efficiency (e.o.p.) determined from the observed number of plaques (column 11) and the actual amount of RNA assayed as determined from columns 6 and 14. Column 14 is determined from assays of acid-precipitable radioactivity on 20 μl aliquots of the final product as compared with column 5.

			Formation of RNA			Concentration of original template			Formation of IU			
1 Transfer No.	2 Interval	3 Time	4 CPM × 10^{-3}	5 Total	6 Δ	7 Σ	8	9 Strands	10 IU	11 Δ × 10^{-5}	12 Σ × 10^{-5}	13 Observed e.o.p. × 10^{-7}	14 Recovery of ^{32}P-RNA
	min			μg	μg		μg						%
0	0	0	0	0.2	0	0	2.0×10^{-1}	1.2×10^{11}	6.0×10^{4}	1.0	1.0	5.5	
1	40	40	64	3.2	3.0	3.0	2.0×10^{-1}	1.2×10^{11}	6.0×10^{4}	5.2	5.2	3.2	54.2
2	40	80	84	4.2	3.6	6.6	4.0×10^{-2}	2.4×10^{10}	1.2×10^{4}	2.2	6.5	2.0	88.3
3	40	120	112	5.7	4.9	11.5	6.7×10^{-3}	4.0×10^{9}	2.0×10^{3}	11.3	17.4	5.3	59.9
4	40	160	134	6.7	5.6	17.1	1.1×10^{-3}	6.6×10^{8}	3.3×10^{2}	5.7	21.2	3.0	42.3
5	30	190	113	5.7	4.4	21.5	1.9×10^{-4}	1.1×10^{8}	55	7.4	27.6	3.0	63.4
6	30	220	144	7.2	6.1	27.6	3.1×10^{-5}	1.8×10^{7}	9	15.0	36.4	3.7	82.4
7	30	250	150	7.5	6.1	33.7	5.1×10^{-6}	3.0×10^{6}	1.5	13.4	48.1	5.0	52.9
8	30	280	162	8.1	6.6	40.3	8.6×10^{-7}	5.0×10^{5}	<1	8.8	54.7	5.2	51.4
9	30	310	164	8.2	6.6	46.9	1.4×10^{-7}	8.4×10^{3}	<1	5.6	58.4	2.0	92.6
10	30	340	156	7.8	6.2	53.1	2.4×10^{-8}	1.4×10^{3}	<1	9.3	66.8	4.0	54.2
11	20	360	134	6.7	5.1	58.2	4.0×10^{-9}	2.3×10^{2}	<1	6.3	73.7	3.7	74.3
12	20	380	121	6.0	4.7	62.9	6.6×10^{-10}	3.8×10^{1}	<1	6.9	84.3	7.0	46.8
13	20	400	123	6.1	4.9	67.8	1.1×10^{-10}	6	<1	3.6	89.4	4.0	49.2
14	20	420	118	5.9	4.7	72.5	1.8×10^{-11}	1	<1	10.8	102.0	5.7	79.7
15	20	440	75	3.6	2.4	74.9	3.1×10^{-12}	0.16	<1	3.2	105.0	5.5	65.4

steepness of the cesium sulfate gradient makes it difficult to achieve a separation clean enough to be completely satisfying.

There exists, however, another approach which bypasses these technical difficulties by taking advantage of the biology of the situation and the fact that we are dealing with a presumed self-propagating entity. Consider a series of tubes each containing 0.25 ml of the standard reaction mixture, but no

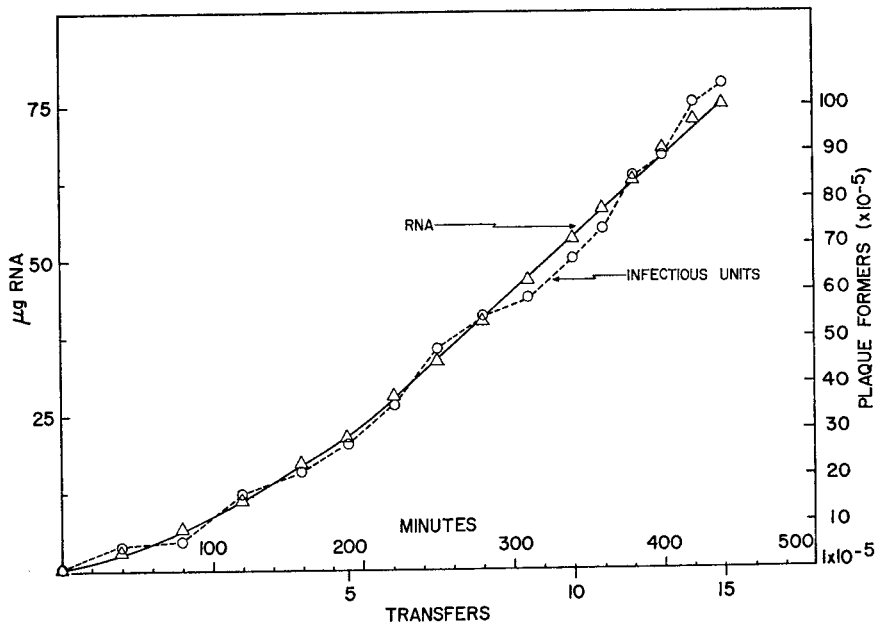

FIGURE 13. RNA synthesis and formation of infectious units in a serial transfer experiment. All details are as described in the heading to Table V and the data are taken from columns 7 and 12 and plotted against elapsed time (column 3) and corresponding transfer number (column 1). Both ordinates refer to amounts found in 0.25 ml aliquots (Spiegelman et al., 48).

added template. The first tube is seeded with 0.2 μg of Qβ RNA and incubated for a period adequate for the synthesis of several micrograms of radioactive RNA. An aliquot (50 μl) is then transferred to the second tube which is in turn permitted to synthesize about the same amount of RNA, a portion of which is again transferred to a third tube, and so on.

If each successive synthesis produces RNA which can serve to initiate the next one, the experiment can be continued indefinitely, and in particular until the point is reached at which the initial RNA of tube one has been diluted to an insignificant level. In fact, enough transfers can be made to insure that the last tube contains less than one strand of the input primer. *If, in all the tubes including the last one, the number of infectious units corresponds to the amount of radioactive RNA found, convincing evidence is offered that the newly synthesized RNA is infectious.*

Table V records a complete account of such a serial transfer experiment and the corresponding legend provides the details necessary to follow the assays and calculations. Sixteen tubes are involved, the first (tube 0) being an unincubated 0 time control. It will be noted that the successive dilutions were such (1–6) that by the eighth tube, there was less than one infectious unit ascribable to the initiating 0.2 μg of RNA. Nevertheless, this same tube showed 8.8×10^5 newly synthesized infectious units during the 30 min of its incubation. Finally, tube 15 which contained less than one strand of the original input produced 1.4×10^{12} new strands and 3.2×10^5 infectious units in 20 min. It should be noted that a control tube lacking added RNA was incubated

TABLE VI

SEROLOGICAL BEHAVIOR OF VIRUS FORMED IN RESPONSE TO "SYNTHETIC" RNA

(Spiegelman et al., 48)

In all cases, lysates were made from *E. coli* Q13, which was also the assay organism. Antisera were used at 1/100 dilution and the incubation temperature was 35°C. The numbers represent plaque formers per milliliter.

	Anti-Qβ			Anti-MS-2		
Antisera virus	0 time	10 min	Survivors, %	0 time	10 min	Survivors, %
Authentic Qβ	1.9×10^8	1.0×10^5	0.052	1.1×10^8	1.06×10^8	96
Virus from synthetic RNA	1.5×10^8	8.8×10^4	0.053	1.5×10^8	1.40×10^8	93

for 60 min. As compared with tube 1 which incorporated 4,800 CPM for each 20 μl in 40 min, the control showed no increase above the 0 time level of incorporated label. Further, no synthesis of infectious units was observed in such controls.

Fig. 13 compares cumulative increments with time in newly synthesized RNA (column 7) and infectious units (column 12). The agreement between the increments in synthesized RNA and newly appearing infectious units is excellent at every stage of the serial transfer and continues to the last tube. Long after the initial RNA has been diluted to insignificant levels, the RNA from one tube serves to initiate synthesis in the next. Further, it may be seen from the comparative constancy of the infective efficiency (Fig. 13 and column 13 of Table V) that the new RNA is fully as competent as the original viral RNA to program the synthesis of viral particles in spheroplasts.

To complete the proof, it was necessary to show that the viruses produced by the synthesized RNA were indeed Qβ, the original source of the RNA used as a seed in tube 1 to initiate the transfer experiment. Since Qβ is a unique serological type, this characteristic was chosen as a convenient diagnostic test. Plaques induced by the RNA synthesized in tube 15 were used to produce

lysates, and the resulting particles exposed to antiserum against MS-2 and Qβ. The results briefly summarized in Table VI show clearly that the synthetic RNA induces virus particles of the same serological type as authentic Qβ.

One perhaps might have imagined that an enzyme carrying out a complex copying process would show a high error frequency when functioning in the unfamiliar environment provided by the enzymologist. Had this been a quantitatively significant complication, biologically inactive strands should have accumulated as the synthesis progressed. That this is not the case is rather dramatically illustrated by the serial transfer experiment (Table V and Fig. 13). The RNA synthesized after the fifteenth transfer is as competent biologically as the initiating natural material derived from virus particles.

The experiments just described demonstrate that the reaction being studied is in fact generating replicas. It would appear that a system has finally been discovered which permits the unambiguous analysis of the molecular basis underlying the replication of a self-propagating nucleic acid. Every step and component necessary to complete the replication must be represented in the reaction mixture described.

K. RECOGNITION OF SIZE AND SEQUENCE

We now focus attention on the discriminating selectivity displayed by the two replicases in their response to added RNA. This is a phenomenon of interest in itself, since it presents us with a system illustrating one of the central unresolved issues of modern molecular biology, viz.; the basis of specific interaction between a protein and a nucleic acid. More immediately, an understanding of this phenomenon is a necessary prerequisite to the design of adequate experiments aimed at the mechanism of replicase action.

An obvious device to explain the specificity exhibited would invoke the recognition of a beginning sequence, a possibility open to the simple test of challenging the replicase with fragmented preparations of homologous RNA. If the initial sequence is the sole requirement, RNA fragmented to half and quarter pieces should serve adequately as templates. One would expect that the initial rate with fragments would be the same as with intact templates, although the reaction might terminate sooner.

Fragments of Qβ RNA are readily obtained and fractionated for size on sucrose gradients. The sedimentation profiles of three such are shown in the inset of Fig. 14. The first is the intact viral RNA (28S), the second a half-piece with a mean of about 17S, and the third possesses a sedimentation coefficient of 7S. The responses of replicase to the three RNA preparations are shown in Fig. 14. It is obvious that the fragmented material is unable to stimulate the replicase to anywhere near its full activity no matter how much is added. The half-piece achieves approximately 10% the rate of the intact strand, whereas the rate with the quarter-piece is only 2% of normal.

In the course of examining template specificity and size requirements of the replicase (49), a striking effect of manganese was observed, which is relevant to the conditions required to examine the mechanism of replication. A typical set

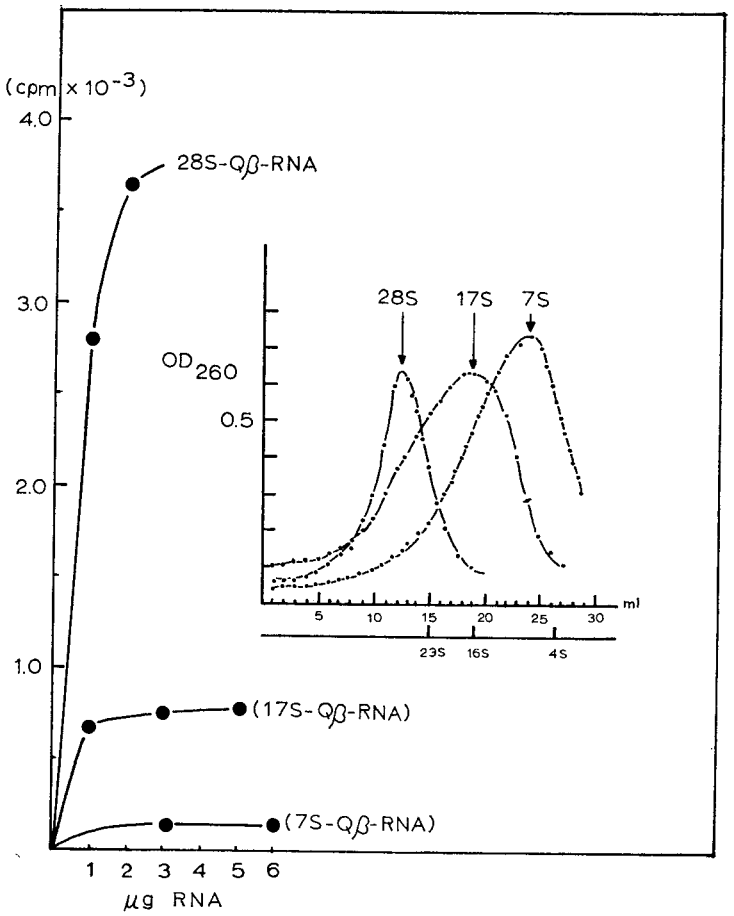

FIGURE 14. Response of replicase to intact and fragmented Qβ-RNA. Inset represents distribution in a linear sucrose gradient (2.5 to 15%) of RNA prepared and fractionated as described in Fig. 9. Each preparation was run in a separate bucket for 12 hr at 25,000 RPM and 4°C in a SW-25 Spinco rotor with markers (Ribosomal RNA) to permit S-value determinations. The template function of each size class of RNA was determined in the standard assay as described in Fig. 7 except that the reaction mixture contained 0.2 μmole of $MnCl_2$ (Haruna and Spiegelman, 49).

of results is summarized in Table VII. In the presence of magnesium the ability of either the 17S fragment or TYMV RNA to stimulate the enzyme is almost negligible, corresponding to 2 and 4% respectively of that observed with intact Qβ-RNA. On the other hand, if Mn^{++} is substituted for Mg^{++} normal synthesis with 28S RNA is strongly inhibited whereas the abnormal

activities observed with 17S and TYMV RNA are stimulated. Indeed, if one were unaware of the optimal conditions for the functioning of this enzyme (Mg^{++} and intact templates), one might be tempted to conclude from the results shown in Table VII that the Qβ replicase prefers TYMV RNA as a

TABLE VII

EFFECTS OF Mg AND Mn ON TEMPLATE SPECIFICITY OF REPLICASE

(Haruna and Spiegelman, 49)

Reaction mixtures (0.25 ml) contained 1 μg of the indicated RNA and 40 μg of protein in addition to the other components detailed in the legend for Fig. 7 with the modifications noted in the body of the table. Incubations were carried out at 35°C for 20 min; washing and counting were the same as described in Fig. 7. TYMV refers to intact RNA isolated from turnip yellow mosaic virus. Qβ-28S is the intact RNA of the Qβ bacteriophage and 17S is the fragment of half-size.

Template	Mg^{++}	Mn^{++}	Incorporated CPM
	μM	μM	
Qβ-28S	—	—	146
Qβ-17S	—	—	63
TYMV	—	—	0
—	—	—	16
Qβ-28S	4	—	4655
Qβ-17S	4	—	109
TYMV	4	—	218
—	4	—	1
Qβ-28S	—	0.12	474
Qβ-17S	—	0.12	222
TYMV	—	0.12	766
—	—	0.12	103
Qβ-28S	3	0.2	2804
Qβ-17S	3	0.2	240
TYMV	3	0.2	1037
—	3	0.2	103

template and that it is comparatively indifferent to the size of the RNA with which it functions.

The abnormalities induced in the reaction by either fragmented RNA or the presence of Mn^{++} are even more obious from the kinetics of the reaction (Fig. 15). As has been observed previously, synthesis continues linearly for extensive periods in the presence of saturating amounts of intact template and Mg^{++}. If, however, Mn^{++} is included in the reaction mixture, the reaction stops in about 60 min even with 28S RNA as a template. If we now examine

the response of the enzyme to fragmented template, it is seen in Fig. 15 that the initial reaction rate is less than 10% of normal and furthermore ceases completely in 30 min whether Mn^{++} is present or not.

The inability of the replicase properly to employ fragments of its own genome as templates argues against a recognition mechanism involving only a beginning sequence. The enzyme can apparently sense when it is confronted with an intact RNA molecule implying that some element of secondary

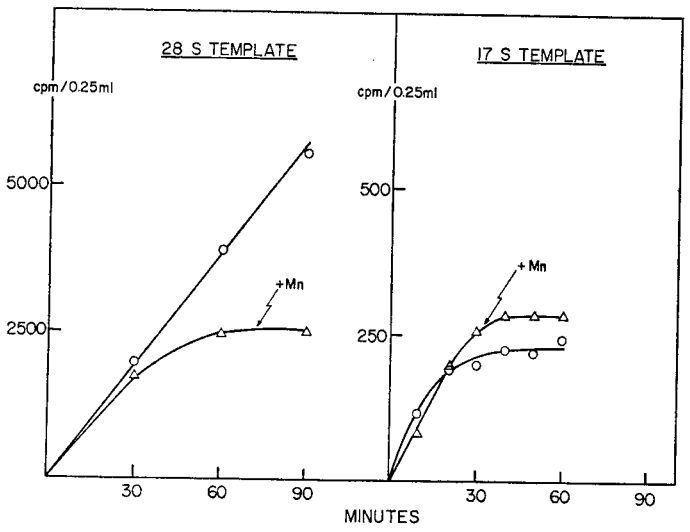

FIGURE 15. Effects of template size and Mn^{++} on kinetics of RNA synthesis. Reactions were set up as detailed in Fig. 14, except that Mn^{++} was omitted or included as indicated. Samples of 0.25 ml were taken and treated for counting as in Fig. 7. Template concentrations were in all cases 1 μg of RNA for each 40 μg of enzyme in 0.25 ml. Note that the ordinate of the right half is $\frac{1}{10}$th that of the left half of the figure (Haruna and Spiegelman, 49).

structure is involved. A plausible formal explanation can be proposed in terms of "functional circularity." Thus, a decision on the intactness of a linear heteropolymer can be readily made by examining both ends for the proper sequences. An examination of this sort would be physically aided by forming a circle using terminal sequences of overlapping complementarity. The enzyme could then recognize the resulting region of double strands. This particular model is offered only as an example of how an enzyme could simultaneously distinguish both sequence and intactness. Whatever the details turn out to be, it appears that the RNA replicases are designed to minimize the futility of replicating either foreign sequences or incomplete copies of their own genome.

L. PROSPECTS FOR THE RESOLUTION OF THE RNA REPLICATING MECHANISM

We may conclude this discussion with an assessment of the current status of the RNA replication problem and an indication of the direction of our present efforts.

It must be emphasized that the doubts raised (section E) about the ribonuclease resistant structures (RS) concern only their function. The structures are real and their existence must ultimately be explained. Certain quantitative features of the time, kinetics of appearance, and proportion of input strand involved in "RS" are difficult to reconcile with a model which insists that they intervene between the initial template and final product. On the basis of these and other difficulties we maintain that a decision cannot be made at present on whether the RS are replicative intermediates of unknown structure, or simply nonfunctional artifacts.

The unambiguous analysis of a replicating mechanism demands evidence that the reaction being studied is in fact generating replicas. Ultimately, therefore, proof must be offered that the polynucleotide product contains the information necessary for the production of the corresponding virus particle in a suitable test system. The experiments described demonstrate that this rather rigorous requirement has finally been satisfied.

It should now be possible to study RNA replication in a simple system consisting of purified replicase, template RNA, ribosidetriphosphates, and magnesium. However, this is a necessary condition, not a sufficient one for success. Possession of an enzyme of this sort does not of itself guarantee that any results observed are necessarily relevant to the nature of the replicating reaction. Attempts at the analysis of replicating mechanisms must recognize the implications of the fact that the enzymes involved are likely to be complicated molecules. High levels of complexity provide the flexibility which permits the occurrence of abnormalities, a potentiality which can be accentuated by exposure to either strange environments or unusual components. Thus, in the absence of primer the DNA polymerase eventually initiates the synthesis of an AT-copolymer (50). In the presence of Mn^{++}, the same enzyme will incorporate ribosidetriphosphates into a mixed polymer (51). Analogously, the DNA-dependent RNA polymerase synthesizes polyadenylate if supplied only with ATP, a reaction which is inhibited if the other ribosidetriphosphates are added (52, 53). Again if presented with a single-stranded DNA, the transcriptase synthesizes a DNA-RNA hybrid (54–57) and if the template is RNA, a duplex RNA results.

The fact that such variations from the norm can occur makes it difficult to draw incontestable conclusions from the appearance of a product in a reaction. Thus, for example, as will be detailed elsewhere, replicase makes a RNAase-resistant structure in the presence of Mn^{++}. The same result occurs if replicase

is presented with either fragments of its own genome or intact heterologous RNA. We recognize that the abnormal has often been fruitfully used in the study of the normal and that even artifacts can ultimately serve to illuminate the reaction in which they are generated. However, it is first necessary to identify the normal. We insist therefore that in the test tube, even more than in the cell, evidence other than mere existence must be provided before a component found is accepted as a normal intermediate in replicated processes.

The study of the normal functioning of the replicases described here requires intact homologous RNA and the avoidance of Mn^{++}. Furthermore, even under optimal conditions, as we know them, prolonged functioning of these enzymes in the enzymologist's test tube can create the possibility of accumulating abnormalities.

Since the enzyme reaction described here does in fact produce RNA strands biologically indistinguishable from the input templates, it should be possible to test all the implications of any proposed mechanism. If two enzymes are required, both must be present and it should be possible either to establish their existence or to prove that one is sufficient. If an intermediate replicating stage intervenes between the template and the identical copy, then these forms should be demonstrably present in the reaction mixture. All experiments designed to test these alternations must be continually monitored to insure that the normal reaction is being followed.

A rather strong negative conclusion can be drawn from the data summarized concerning the possible role of transcriptase as the "second enzyme" for RNA replication, a mechanism some find attractive. The complete absence of detectable transcriptase from our preparation would appear to eliminate it as a participant in RNA replication.

It seems likely that the most telling data are derivable from experiments in which the initiation of chains is synchronized. To begin with, the examination of the product synthesized, prior to the appearance of mature strands, can be compared with the product formed in more extensive synthesis. The use of different isotopes on template (e.g. ^{3}H) and product (e.g. ^{32}P) permits a sensitive search for intermediate complexes between the two.

We may briefly list potentially informative experiments which used these and other devices:

(*a*) A comparison of ribonuclease resistance of product and template at various stages of synthesis.

(*b*) The search for a physical complex between the ^{32}P product and the ^{3}H template in sucrose gradients and in equilibrium density gradients of Cs_2SO_4. In the latter the templates were, in some cases, additionally labeled with ^{15}N to give them a unique density position. Here the early (1 to 5% synthesis) events are most crucial.

(*c*) A detailed analysis of the base composition during the progress of early

synthesis. The resulting data are particularly informative in the case of Qβ, since its A/U ratio is 0.75 and that of its complement is therefore 1.33. The formation of the complement as an initial step is consequently easily detected.

(*d*) Along similar lines, a comparison of nearest neighbor analysis to all four bases in early and late synthesis should reveal whether a complement or the identical copy is being made in the early periods.

(*e*) Finally, the degree of complementarity between the product and the original template at various stages of strand formation can be determined by hybridization tests. In this connection it may be noted that the required annealing experiments are not as simple, either logically or technically, as some recent contributions would suggest.

Virtually, all the experiments listed above have been carried out and the others are in the final stages of completion. The detailed data and conclusions will be recorded elsewhere. Here we may state that thus far, we have found no evidence to encourage the idea that a duplex containing the mature strand and its complement plays a role in replication.

It is important to emphasize that none of this should be taken to mean that our experiments have eliminated the use of complementarity in RNA replication. There are readily designed mechanisms which involve complementarity without requiring the synthesis of the intact complementary strand. An extreme example may be briefly noted: Consider the possibility that a representative of each of the four nucleotides is attached to the enzyme. These could be permanent components or replaceable ones and are used by the enzyme for complementary reading of the template as a guide for building an identical copy. Other mechanisms involving transient partial complements can also be devised.

It seems likely that many of the uncertainties which still exist about RNA replication will yield relatively soon to the proper experiments. We are tempted to end the present discussion with only a slight modification of the conclusion used in an earlier (58) essay on protein synthesis. "The crucial experiments have not yet been executed. However, the systems required for their performance are with us, or close to hand. The outlook is depressingly bright for the quick resolution of *another* interesting problem."

This investigation was supported by United States Public Health Service Research Grant CA-01094 from the National Cancer Institute and National Science Foundation Grant GB-2169.

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Discussion

Dr. Luria: Thank you very much, Dr. Spiegelman. As you were talking, I was thinking that one reason you get very wonderful answers and a very clear way to transmit them to us is because of a habit I discovered today for the first time in the 25 years that we have known each other: instead of asking questions of yourself, you're asking questions of the enzymes. I enjoyed your conversation with the polymerase.

Dr. Charles Weissmann: I would like to make a comment on this matter of double-stranded RNA. Dr. Billeter, Dr. Libonati, and Dr. Viñuela have carried out the following experiment. MS2-infected *E. coli* was pulsed with ^{14}C-guanine at 23 min after infection, which is the time at which viral RNA synthesis is maximal. The pulse was for 8 sec and after that the double-stranded RNA was isolated. Now, the question was, if you examined this double-stranded RNA, where would you find the radioactivity? Would you find it in the plus strand, that is, in the viral type strand or in the complementary or "minus" strand? The analysis showed that the ratio of radioactivity in the plus strand to that in the minus strand was about 6 to 1. If a chase was given after this pulse, then it was found that 90% of the radioactivity in plus strand was displaced from the duplex. This suggests that a complex consisting of a minus strand and a plus strand is an intermediate in synthesis. I do not wish to say that this complex is in fact double-stranded the way it is generally understood, namely, that it is hydrogen-bonded and ribonuclease-resistant. I'm just saying that it is a complex which after isolation is double-stranded; it is therefore possible to characterize it by this criterion.

It seems then that a complementary (or minus) strand is involved in replication and can be demonstrated in this fashion. We have had similar results earlier with RNA synthetase (which is the New York counterpart of replicase) and we found about a year ago that it actually does make replicas of viral RNA, as was shown by annealing techniques. In the in vitro reaction we find that part of these newly formed plus strands are in double-stranded form. Analysis of the double strand shows that it is only the plus strand which is labeled and not the minus, again indicating that the minus strand serves as a template.

Now, I would like to ask you one question. If you look into your reaction mixtures, do you find complementary—not double strands—do you find complementary (i.e., minus) strands if you anneal for them?

Dr. Spiegelman: When we have fragments, we do. When we don't, we don't.

Dr. Weissmann: And, you definitely obtain cases when you do not have any fragments?

Dr. Spiegelman: Right.

Question from the Floor: My question has a little bit to do with the historical development of Dr. Spiegelman's work. Many years ago you got involved in galactose metabolism and at that time you thought in terms of a cytoplasmic gene. Much more recently you've been able to show that all you need for this synthesis of ribosomes is a nucleus and you don't need anything in the cytoplasm for that. And, now you have RNA-dependent synthesis, whether you believe or not that it's the real mechanism for virus, so my question has really to do with the ribosomes. Do you think that there still may be some mechanism where the ribosome material is necessary in the cytoplasm?

Dr. Spiegelman: From about 1946 to 1951 we were studying adaptation to melibiose and galactose in yeast. Certain properties of these systems, particularly "long term adaptation to galactose," led us to propose that the RNA genetic messages had at least limited capacity to replicate in the cytoplasm and we adopted the term "plasmagenes" as the name for these informed genetic intermediaries. For its time it was not a bad hypothesis. While one hates to witness the decease of an attractive idea, I feel that the concept of the plasmagenes as a self-propagating genetic message must be abandoned. There is no evidence I find compelling that leads me now to entertain the idea of the existence of a mechanism in normal cells which generates RNA copies from RNA.

Let me return for a moment to Dr. Weissmann's comment. I must emphasize that I'm not denying the absolute relevance of double-stranded structures to RNA replication. I do maintain that every time we have tested their relevance with our enzyme system we have thus far obtained negative replies. I do not agree with your conclusion that the positive outcome of annealing experiments with minus strands implies the production of positive strands. Fragments representative of either the whole or a part of the 28S molecule would yield the same result.

Our preparations are making complete replicas. The detection methods we used should have revealed an intermediate complex. Nevertheless we can't find them. The only time we detect them is when the enzyme is producing biologically inactive material. That's the fact we have to face.

Dr. Weissmann: You are speaking of double strands and I would like to stress that the matter here in question is the complementary (i.e. minus) strands; that is all that we're really concerned about right now.

Dr. Luria: It seems to me that the most important thing to realize (I am sure Dr. Spiegelman will agree with me since I heard him say it himself yesterday in Cambridge) is that at the moment we should not assume that we have a theory or a model of replication of the RNA of viruses. There are a number of facts and many of these facts can fit into one or another hypothesis, but if you think about it carefully, you'll see that each one of those hypotheses includes some preconception derived from other fields than that of the biosynthesis of the viral RNA. It is perfectly possible, for example, that the viral RNA might have developed one or more polymerases or replicases, some that can be effective in one condition and others that can function

under other conditions. One might conceive, for example, that during cellular revolution, stranded in a world of DNA-controlled replication, some poor RNA remnants of a previous age might sometime have conserved their own ancestral ways and at the same time may also have taken advantage of the new opportunities and developed other mechanisms more acceptable to a Watson-Crick world.

So, I think that the most important thing to do when one faces something so revolutionary as an RNA virus is to keep one's mind open and one's Heart Association functioning.

Proteins don't have any such luck as nucleic acids. Proteins cannot be copied, at least as far as the present dogma goes. Of course, dogmas may break down, but for the time being I think we are confident that once we leave nucleic acid we are dead. At least, we don't reproduce anymore.

In the problem of protein biosynthesis there are a great many things we do understand and there are also many things which are still obscure. One of the things that impresses me most about this field is that new approaches are continuously being developed. One of the reasons that I asked Dr. Lengyel to be a speaker here today is that, in the one year that we spent together in Paris, I was impressed by the way he was exploring all sorts of biological approaches that could contribute to the study of protein biosynthesis. Dr. Lengyel is going to talk about "Problems in Protein Biosynthesis."